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Original Article

Overexpression of CRM1 indicates poor prognosis and its knockdown suppresses tumor growth in gastric cancer

Yandong Li[1*], Haixiao Wang[2*], Qun Li[1], Qiang Li[1], Yong Gao[1]

[1]*Department of Oncology and Hematology, Shanghai East Hospital, Tongji University School of Medicine, 150 Ji-Mo Road, Shanghai 200120, China;* [2]*Department of General Surgery, Huai'an First People's Hospital, Nanjing Medical University, Huai'an 223001, Jiangsu, China.* [*]*Equal contributors.*

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Abstract: Chromosome maintenance region 1 (CRM1), known as one of major nuclear exporters, plays a critical role in transmission of proteins and RNAs from nuclear to cytoplasm. Increasing studies have demonstrated that overexpression of CRM1 contributes to tumorigenesis and is closely related to tumor development and progression. However, the expression pattern, clinical significance and function of CRM1 in human gastric cancer have not been well characterized to date. In this study, we investigated the expression of CRM1 in 22 pairs of primary gastric cancer samples by quantitative real-time PCR and additional 71 gastric cancer samples by immunohistochemical staining on a tissue array. The results indicated that CRM1 was frequently upregulated in gastric cancer tissues and this upregulation was associated with tumor stage, tumor size, lymph node metastasis and distant metastasis of patients. Moreover, the patients with high expression of CRM1 were inclined to have shorter survival time than those with low expression of CRM1. Functionally, knockdown of CRM1 by RNA interference significantly inhibited the growth of gastric cancer cells in vitro and in vivo. In addition, the effect of downregulation of CRM1 on gastric cancer cells promoted cell death and altered some apoptosis- or cell cycle-related gene expression. Taken together, these findings suggest that CRM1 may facilitate to gastric cancer progression and serves as an indicator for poor prognosis and a potential therapeutic target for anticancer treatment.

Keywords: CRM1, cell proliferation, gastric cancer, poor prognosis

Introduction

Gastric cancer is the second most common cause of cancer-related deaths worldwide, especially in developing countries [1]. The primary treatments of gastric cancer are surgical resection, conventional chemotherapy or radiotherapy. Although surgical techniques and anticancer drug therapies have made great progress in recent years, advanced gastric cancer is still the main cause of death and strongly associated with poor prognosis. Therefore, it is necessary to identify some suitable biomarkers for early diagnosis and predicting prognosis for gastric cancer patients, as well as new and effective molecular targets controlling gastric cancer progression to improve prognosis and therapeutic strategies.

Chromosomal region maintenance 1 (CRM1), a member of the karyopherin b superfamily of transport receptors, mediates rapid nuclear exit of proteins and RNAs to the cytoplasm [2, 3]. It is known that protein shuttling between the nucleus and the cytoplasm plays an important role in cell proliferation and survival. A number of cell cycle- or cell death-related factors, such as p53, p27, HDAC5, SP1 and EGFR, are CRM1-dependent during nuclear export [4-8]. Indeed, dysregulation of CRM1 frequently results in various diseases including cancer. Many reports have shown that CRM1 is abundantly expressed in a wide range of human cancers. Increased expression of CRM1 is also associated with poor overall survival and progression of multiple cancers, including ovarian cancer, pancreatic cancer, cervical cancer, glio-

ma and myeloma [5, 9-12]. However to date, little is known about CRM1 function and clinicopathological significance in gastric cancer.

In the present study, we evaluated the correlation between the expression of CRM1 and the clinical characters of human gastric cancer patients. Furthermore, we also investigated its potential roles of CRM1 knockdown on cell growth of gastric cancer cell in vivo and in vitro. In addition, mechanistic analyses revealed that CRM1 knockdown leads to cell death and some related proteins reduction. Our findings suggest that CRM1 expression could be used as a good prognostic factor for the gastric cancer patients and an effective molecular target for anticancer treatment.

Materials and methods

Human tissue samples and ethics statement

A total of 22 paired cancerous and matched adjacent noncancerous gastric mucosa tissues were collected from gastric cancer patients undergoing gastrectomy at Shanghai East Hospital, and the diagnosis was confirmed by pathological examination. After surgical resection, fresh tissues were immediately immerged in RNA later (Ambion) to avoid RNA degradation, stored at 4°C overnight to allow thorough penetration of RNA later into the tissue and then frozen at -80°C until RNA and protein extraction was performed. A tissue array with71 paired of paraffin-embedded primary gastric carcinoma tissues, which had been collected between 2007 and 2008, were obtained from Shanghai Outdo Bioteck (HStm-Ade167Sur-01). The follow-up data of the gastric cancer patients in this study are available and complete. The histopathological type and stage of gastric cancer were determined according to the criteria of the World Health Organization classification and the TNM stage set out by the Union for International Cancer Control. All the researches were approved by the Ethics Committee of Shanghai East Hospital, Tongji University School of Medicine, and written informed consent was obtained from each patient involved in this study.

Immunohistochemistry

Immunohistochemical staining was performed as previously described [13]. The tissue microarray slides were incubated with primary antibodies against CRM1 (1:200; Santa Cruz, CA, USA) overnight at 4°C. The slides were subsequently incubated with the secondary antibody for 30 min at room temperature and then developed with the 3,3'-diaminobenzidine (DAB) solution followed by counter staining with hematoxylin. Each section was scored according to the intensity of labelling, from 0 (no staining) to 3 (strong staining). The degree of immunohistochemical staining was evaluated independently by two pathologists.

Cell lines and culture conditions

The gastric cancer cell lines, BGC823 and AGS were obtained from the Committee of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cell lines were cultured in MEM media supplied with 10% heat-inactive fetal bovine serum (FBS). The cells were incubated at 37°C in a humidified chamber containing 5% CO_2.

Extraction of total RNA and quantitative real-time PCR

Total RNA was extracted from the tissue samples using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's protocol. Total RNA concentration was assessed by measuring absorbance at 260 nm using a NANO DROP spectrophotometer (ND-1000, Thermo Scientific, USA). Reverse transcription (RT) to synthesize the first-strand of cDNA was performed with 2 μg of total RNA treated with M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's recommendations. The resulting cDNA was then subjected to real-time quantitative PCR for evaluation of the relative mRNA levels of CRM1 and β-actin. The following primers were used to specially amplify the *CRM1* and β-actin. Forward: 5'-CAACTAAAGCAGATGCTTCC-3' and reverse: 5'-TAATGAAGGGCCTCCATAAG-3' for *CRM1*; Forward: 5'-AGAGCCTCGCCTTTGCCGATCC-3' and reverse: 5'-CTGGGCCTCGTCGCCCACATA-3' for β-actin. Quantitative real-time PCR was performed with ABI 7500 Real Time System and SYBER green reagent (TaKaRa, Japan). The mRNA level of each sample was normalized to β-actin prior to comparative analysis using $2^{-\Delta Ct}$ method.

RNA interference

For knockdown of CRM1 expression, CRM1-specific small interference RNAs (siRNAs) were chemically synthesized (GenePharma, Shang-

hai, China). The sense sequences of si-CRM1 are: si-1 5'-CUCAGAAUAUGAAUACGAAdTdT-3'; si-2 5'-GAAUGUUCCAAUGUUUCGAdTdT-3'. The irrelevant nucleotides not targeting any annotated human genes was used as negative control (si-NC). Its sense sequence: 5'-UUCUCCGAACGUGUCACGUdTdT-3'. Cell transfection with siRNAs was conducted using Lipofectamine 2000 (Invitrogen, USA) in accordance with the manufacturer's instructions.

Western blot analysis

The homogenized gastric cancer samples, including tumor and non-tumor tissues, as well as cell lines, were lysed in RIPA lysis buffer, and the lysates were harvested by centrifugation (12,000 rpm) at 4°C for 30 min. Approximately 100 ng protein samples were then separated by electrophoresis in a 8% sodium dodecyl sulfate polyacrylamide gel and transferred onto nitrocellulose membranes. After blocking the non-specific binding sites for 60 min with 5% non-fat milk, the membranes were incubated overnight at 4°C with antibodies against CRM1, p53, or β-actin (Santa Cruz, CA, USA), against p21, BCL2 and cleaved-PARP (Cell Signalling Technology, USA). The membranes were then washed three times with TBST (Tris-buffered saline with tween-20) for 15 min and probed with anIRDye800-conjugated secondary antibody (1:1,000, Rockland, USA) for 1 h at room temperature. After three washes, the signals were visualized with the Odyssey infrared imaging system (LI-COR, USA).

Cell viability assay

For cell viability assay, gastric cancer cells ($3\sim4\times10^3$ per well) were seeded into 96-well plates in 100 μl culture medium. Cell Counting Kit-8 (Dojindo Laboratories, Japan) was used to measure the absorbance value of OD 450 at 24 h intervals for 5-7 days. All experiments were independently repeated at least three times.

Soft agar growth assay

Gastric cancer cells were suspended in the media containing 0.4% agar and overlaid on 1% agar in 24-well plates (1000 cells/well). After 2-3 weeks, colonies were counted and photographed. All experiments were independently repeated at least three times.

Cell death analysis

Cell death rates were measured by flow cytometry analysis of cells stained with propidium iodide (PI) only. Briefly, forty 48 h after transfection with siRNAs, gastric cancer cells were collected and then approximately 0.5×10^6 cells were resuspended in 500 μl of PI solution (20 μg/ml PI in phosphate buffered saline) at 4°C for 30 min. A total of 10^4 cells were analyzed for each sample. PI positive cells were detected and analyzed by a FACS Calibur instrument (Becton-Dickinson, USA) and CellQuest software (Becton-Dickinson, USA).

Tumourigenicity assay in nude mice

1.5×10^6 of BGC823 cells stably expressing shCRM1 or shNC were injected subcutaneously into the flanks of 4-week-old male BALB/c nude mice (n=6, SLAC Laboratories, Shanghai, China). The tumours dimensions were measured twice a week using a digital calliper and the tumours volume was calculated using the formula: $V=0.5\times \text{width}^2 \times \text{length}$. After 4 weeks, the mice were sacrificed and the tumors were dissected and photographed. All animal handling and experimental procedures were approved by the Ethics Committee of the Shanghai East Hospital, Tongji University.

Statistical analysis

The χ^2 test was used to analyze the relationships between CRM1 expression and various clinicopathological parameters. Survival curves were calculated using the Kaplan-Meier method and compared by the log-rank test. Results obtained from in vitro and in vivo experiments were presented as mean ± standard deviation (SD) and analyzed by two-tailed Student's *t*-test. $P<0.05$ was taken to indicate statistical significance.

Results

Increased CRM1 expression in gastric cancer samples

The mRNA level of CRM1 was determined by quantitative real-time PCR (qRT-PCR) assays in 22 paired cancerous and the matched adjacent normal gastric mucosa tissues. The results showed CRM1 mRNA was significantly higher in 12 (54.5%) cancer bearing tissues

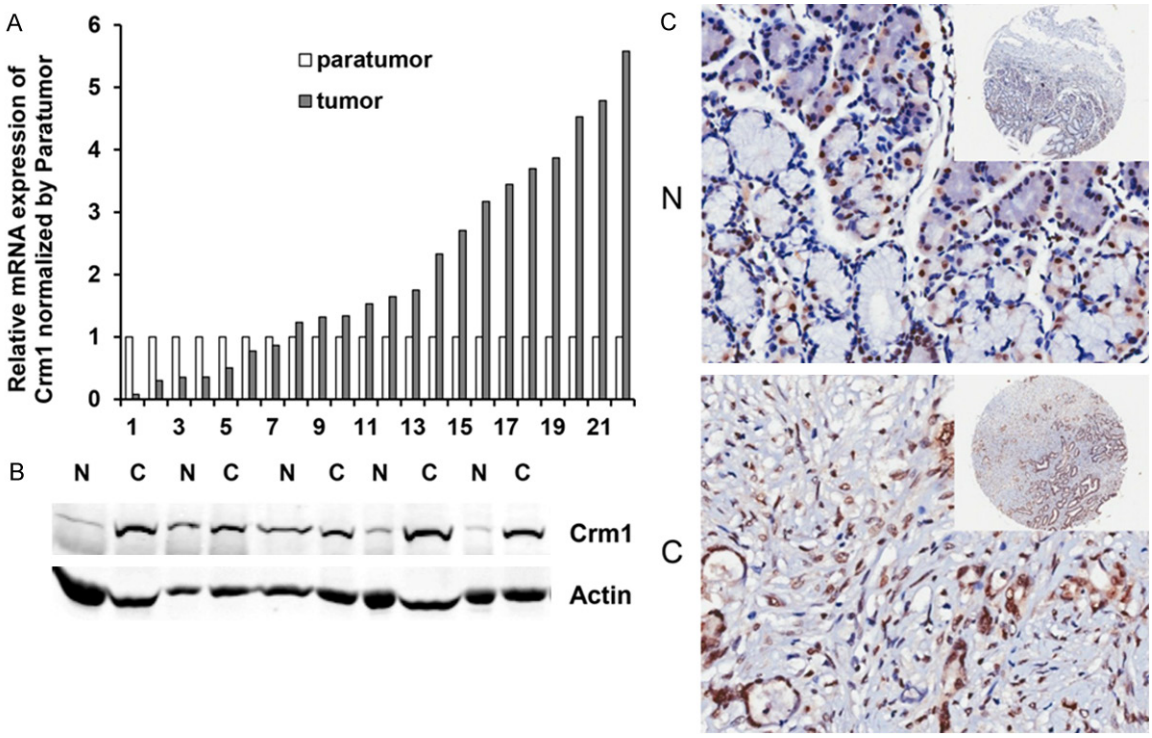

Figure 1. Expression pattern of CRM1 in gastric cancer samples. A. The expression of CRM1 in 22 pairs of gastric cancer specimens and non-cancer tissues was measured by qRT-PCR. C, gastric cancer tissues; N, adjacent non-cancer tissues. B. Protein expression of CRM1 was measured in 5 paired of representative gastric cancer samples by Western blotting. C. Representative immunohistochemical staining of gastric cancer samples with an anti-CRM1 antibody on a tissue array.

compared with the adjacent non-cancer tissues (C/N>1.5 fold, **Figure 1A**). The protein level of CRM1 in 5 paired representative gastric cancer specimens were detected by western blot analysis. As shown in **Figure 1B**, CRM1 protein expression was markedly overexpressed in tumor tissues consistent with the qRT-PCR results. Furthermore, we performed immunohistochemical staining on a tissue array containing 71 pairs of gastric cancer specimens with an anti-CRM1 antibody. As expected, CRM1 levels were upregulated in 36 (50.7%) of the 71 HCC specimens (**Figure 1C**). Collectively, these data demonstrated that CRM1 is frequently overexpressed in gastric cancer.

Upregulation of CRM1 implicates cancer progression and poor prognosis

In order to evaluate the role of CRM1 in gastric cancer tissues, we analyzed the correlation between CRM1 expression and clinicopathological characteristics in the 71 patients on the tissue array. The results of the analysis revealed that CRM1 upregulation was significantly associated with tumor stage, tumor size, lymph node metastasis and distant metastasis ($P<0.05$, **Table 1**). Other clinicopathological parameters, such as patient gender, age, lymphatic invasion and tumor differentiation were not significantly related to the upregulation of CRM1 ($P>0.05$, **Table 1**). More importantly, when we assessed the association between CRM1 expression and patients' survival using Kaplan-Meier analysis with the log-rank test, we found those patients with CRM1 high expression had a shorter overall survival time than those patients with CRM1 low expression ($P<0.05$, **Figure 2**). These results suggested that increased CRM1 expression may enhance cancer cell proliferation, tumor progression and indicate poor prognosis.

Downregulation of CRM1 suppresses gastric cancer cell growth

To evaluate the effects of CRM1 on cell proliferation, RNA interference against CRM1 was carried out in two gastric cancer cell lines: AGS and BGC823. CRM1 expression in transfected

Table 1. Expression of Crm1 protein in human gastric cancer according to clinicopathological features of patients

Clinicopathological variables	Crm1 expression		*P*-value
	T>N (n=36)	T≤N (n=35)	
Age (year)			
<60	12	15	
≥ 60	24	20	0.409
Gender			
Male	23	25	
Female	13	10	0.497
Tumor Size (cm)			
≤ 3	4	13	
>3	32	22	0.010*
Lymph node metastasis			
n0	4	11	
n1-n3	32	24	0.036*
Lymphatic invasion			
Present	7	5	
Absent	29	30	0.562
Tumor differentiation			
I, II	9	11	
III, IV	27	24	0.547
Distant metastasis			
M0	28	34	
M1, M2, M3	8	1	0.036*
TNM stage			
I, II	8	17	
III, IV	28	18	0.020*

*P<0.05 statistically significant difference.

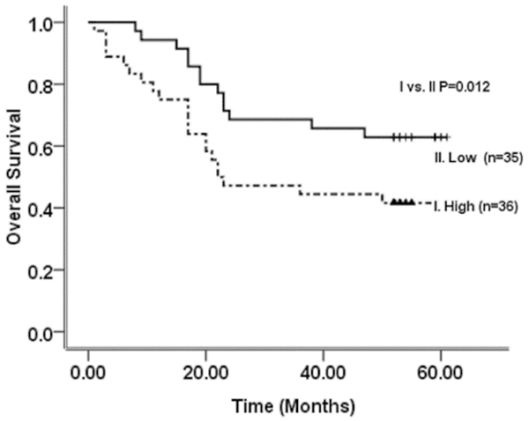

Figure 2. Increased CRM1 expression predicts an unfavorable prognosis. The association between patient survival and CRM1 expression was estimated using the Kaplan-Meier method and the log-rank test (P<0.05).

cells were detected by Western blotting (**Figure 3A**), indicating the CRM1 specific siRNAs, siCrm1-1 and siCrm1-2 exhibited an efficient knockdown of CRM1 relative to the control si-NC. The following cell growth assay by CCK8 method revealed that cell growth rate in CRM1 specific siRNA transfected AGS and BGC823 cells were significantly lower than si-NC transfected control cells (**Figure 3B**). Furthermore, the ability of AGS and BGC823 cells forming colonies in soft agar was also explored. This ability could reflect tumor cell malignancy. As shown in **Figure 3C**, the cells transfected with CRM1 siRNAs formed much fewer colonies in both cell lines than those transfected with si-NC, suggesting CRM1 is required for gastric cancer cell growth in soft agar. These results demonstrated that targeting CRM1 by siRNA significantly inhibits gastric cancer cell growth.

Decreased CRM1 expression induces gastric cancer cell death

To address whether CRM1 affects gastric cancer cell death, we used PI (Propidium Iodide) to stain dead cells or advanced apoptotic cells, since PI cannot enter into cell inside of live cells or early apoptotic cells. 48 h after transfection of CRM1 siRNAs or control siRNA, cells were harvested and stained with PI for 30 min. Cell death rates were quantified through flow cytometry analysis. As shown in **Figure 4**, AGS and BGC823 cells with decreased CRM1 expression have higher cell death rate than control cells, respectively. This result implicated that CRM1 is required for cell survival in gastric cancer cells.

Downregulation of CRM1 reduced the tumorigenicity of gastric cancer cells in vivo

To further determine the effect of CRM1 on gastric cancer cell growth and survival in vivo, a tumorigenicity mouse model was employed in this assay. 1.5×10^6 of CRM1-shRNA-transfected BGC823 cells were subcutaneously injected into the flank of BALB/c nude mice while control cells were injected into the opposite side of the same mice (n=6). Consistent with the effect of CRM1 knockdown in vitro, the tumours generated by the cells with CRM1 knockdown were significantly smaller than those of the control group (**Figure 5A**). The two plotted growth curves under observation and the wet weight of

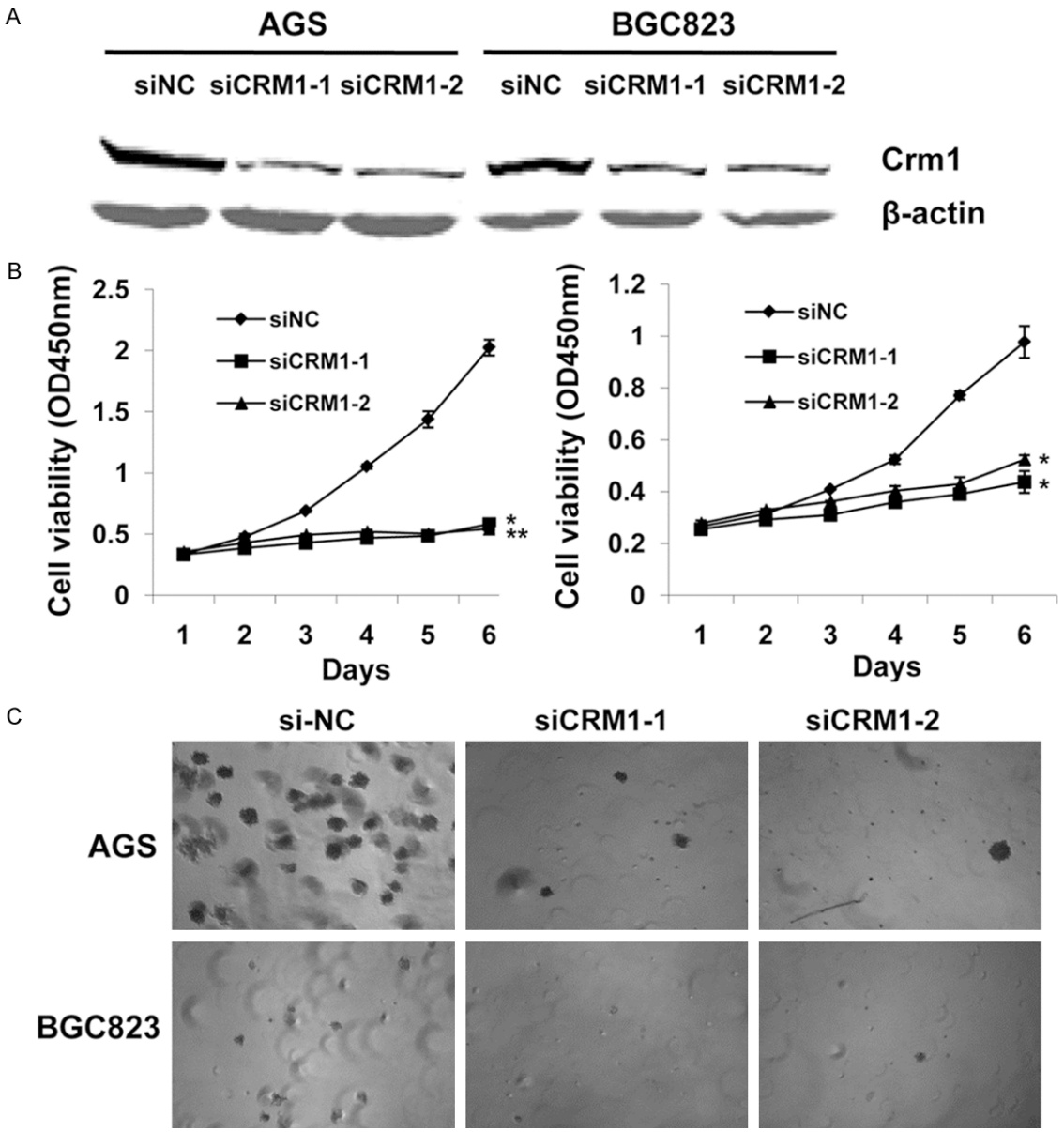

Figure 3. CRM1 is required for gastric cancer cell growth. A. CRM1 knockdown by siCrm-1 and -2 in AGS and BGC823 cells were analyzed by western blot in which an endogenous CRM1 antibody was used to detect CRM1 expression. B. Cell viability assays were performed to explore the effect of CRM1 knockdown on two cells above by CCK8 method. C. Soft agar assays were carried out for the effect of CRM1 knockdown on cells as indicated.

xenograft tumours showed a distinct difference ($P<0.05$, **Figure 5B**, **5C**). These data demonstrated that CRM1 reduction inhibits tumor cell growth in vivo, suggesting CRM1 may serve as a therapeutic target in gastric cancer.

CRM1 knockdown results in upregulation of p53, p21 and cleaved-PARP and downregulation of BCL2

Previous reports have indicated that CRM1 knockdown inhibited cell cycle and induced cell apoptosis [11, 14]. To understand the mechanism by which CRM1 affects gastric cancer progression, we performed Western blot analysis to detect cell death or cell cycle related gene expression in BGC823 cells after transfection with CRM1 siRNAs or control siRNA. The resulting data showed silencing of CRM1 with siCrm1-1 and siCrm1-2 led to an increase in the protein level of p53, p21, and cleaved-PARP; as the same time, the anti-apoptosis protein BCL2 was decreased in the CRM1 knockdown cells (**Figure 6**). These data indicated that CRM1

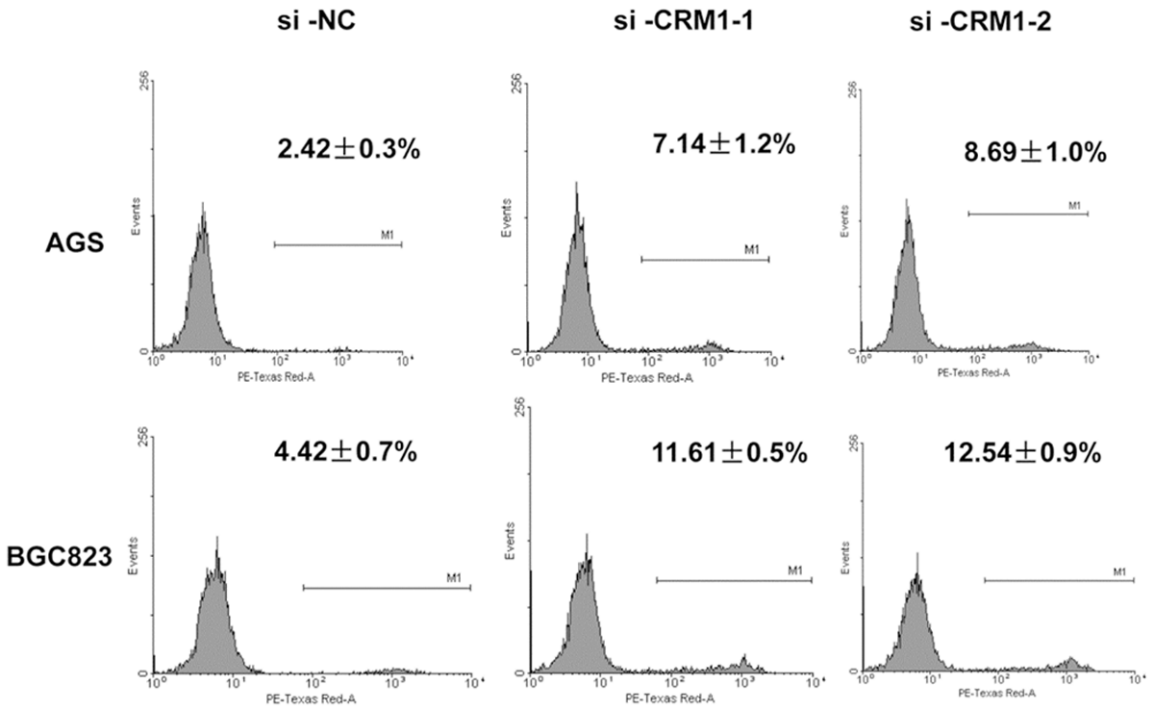

Figure 4. Flow cytometric analysis of AGS and BGC823 cells transfected with si-Crm1 or si-NC. 48 h after transfection, cells were harvested and treated with 20 μg/ml PI solution for 30 min. The percentage of PI positive cells represents the cell death rate.

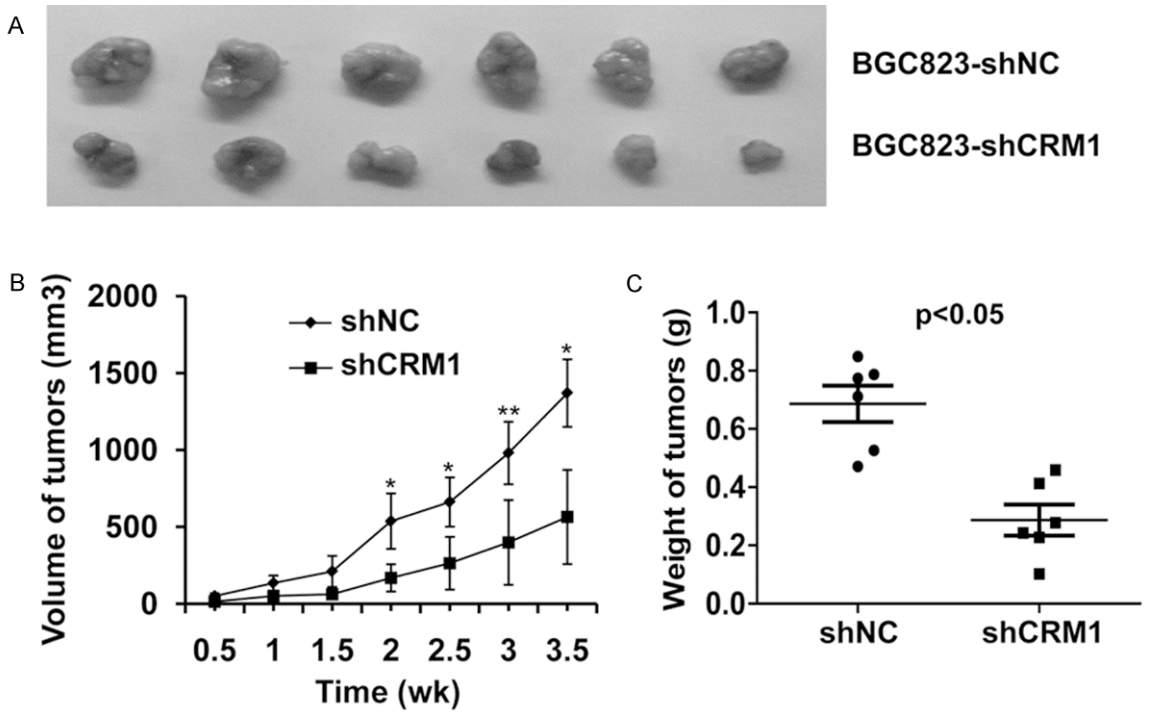

Figure 5. The effect on tumourigenicity of CRM1 knockdown in BGC823 cells. A. The xenograft tumors were removed from tumor-bearing mice (n=6). The photo indicates that the mice injected with control cells or shCrm1 cells; B. Tumor volumes were measured twice a week. Points, mean volume; bars, SD. *, $P<0.05$; **, $P<0.01$; C. Tumours were weighed immediately after removal. Mean tumours weight is exhibited. bars, SD.

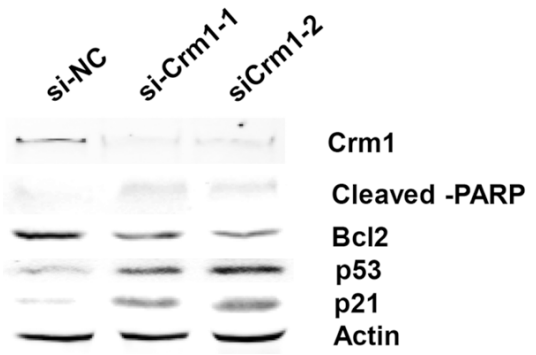

Figure 6. CRM1 knockdown altered the expression of cell cycle-or cell death-related proteins. Western blot analysis indicated the expressions of CRM1, cleaved-PARP, p53, p21 and BCL2 in BGC823 cells, which were transfected with or without siCRM1, siNC, respectively. *β*-actin was used as a loading control.

downregulation plays a negative role in these protein abundances.

Discussion

In spite of great advances in diagnosis and therapy, gastric cancer remains one of the most deadly neoplasms, with an unfavorable outcome after radical gastrectomy [15]. Thus, identification of some suitable biomarkers for early diagnosis and predicting prognosis is of significance for gastric cancer patients. In the present study, we aimed to evaluate the possibility of using CRM1 as a novel marker for the prognosis of gastric cancer patients and to elucidate the possible involvement of CRM1 in gastric cancer development.

We first detected the expression of CRM1 in human gastric cancer specimens by qRT-PCR, western blot and IHC. Our data showed that CRM1 is upregulated in most of gastric cancer tissues compared with adjacent non-cancer tissues, in line with the expression pattern of CRM1 in other cancers. Importantly, CRM1 overexpression was closely associated with tumor stage, tumor size, lymph node metastasis and distant metastasis of patients. As well, there is also a close correlation between CRM1 overexpression and the overall survival time of patients with gastric cancer. These results support the conclusion that CRM1 may serve as a biomarker for monitoring gastric cancer progression and prognosis. However, our study may have some limitations such as region, population, and ethnicity. It is required more clinical data to be supplied in the future. Notably, one previous report has provided evidence supporting our issue using additional clinical samples in different area [16].

Next, we discussed to determine whether CRM1 is involved in gastric cancer development. With the RNA inference technology targeted CRM1, we showed that CRM1 knockdown significantly inhibits gastric cancer cell growth whether in the anchorage dependent- or independent way. In a mouse model, CRM1 knockdown also has a significant inhibitory effect on xenograft tumor growth. These data are the first to demonstrate the roles of CRM1 in gastric cancer cells, suggesting CRM1 may offer a new therapeutic target for this disease. Mechanistically, FACS analysis demonstrated that CRM1 knockdown induced cell death, including advanced cell apoptosis by detection of PI-positive cells. As previously reported in other cancers [5, 11, 17, 18], western blot analysis indicated altered expression of p53, p21, BCL2 and cleaved-PARP toward cell cycle arrest or cell apoptosis in CRM1 knockdown cells. These oncogenic proteins and tumor suppressors may mediate the function of CRM1 in the regulation of tumorigenesis.

Regarding CRM1 inhibitors, Leptomycin B (LMB) was the first specific CRM1 inhibitor to be discovered, which modifies CRM1 at a reactive site cysteine residue (cysteine 529) to block binding of the nuclear export sequence to CRM1 [19]. Although LMB was tested in a phase I clinical trial before, it failed to proceed due to severe toxicities. Additional CRM1 inhibitors have been increasingly isolated or synthesized in recent years [20-22], however, most of them are irreversible inhibitors which have toxicity on normal cells like LMB. Therefore, exploring new inhibitors against CRM1 with reversible and low toxicity is urgent. With the delivery technology development, CRM1 siRNAs are now becoming promising. Our study has provided two effective siRNA sequences for further investigation in future.

In summary, we demonstrated that CRM1is overexpressed in patients with gastric cancer, and has an association between its overexpression and disease progression and poor prognosis. Our findings suggest that Crm1 may have potential as both biomarker and therapeutic target in gastric cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Yong Gao, Department of Oncology, Shanghai East Hospital, Tongji University School of Medicine, 150 Ji-Mo Road, Shanghai 200120, China. Tel: +86-21-61569591; Fax: +86-21-58798999; E-mail: gaoyon@hotmail.com

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